A FE-SEM study on the tobacco leaf epidermis

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The leaf epidermis of *Nicotiana tabacum* L. was studied using high-resolution field emission scanning electron microscopy. Tobacco leaf epidermis specimens were prepared in four different ways. All preparation schedules yielded the same results. The gross morphology of the epidermis shown by FE-SEM is similar to that revealed in TEM work, namely a cuticular membrane and a cell wall. The FE-SEM revealed a globular structure for the cuticular membrane. The cell wall was found to be stratified, revealing microfibrils with a diameter of *ca.* 30 nm which corresponds to those of cellulose microfibrils of cell walls.

Keywords: cellulose microfibril, cell wall, cuticular membrane, cuticle, epidermis, FE-SEM.

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Introduction

The primary aerial parts of higher plants are covered, with the exception of the stomatal openings or glandular apertures, by a continuous extracellular lipid membrane called the cuticle (Chamel *et al.* 1991). The cuticle of plants serves many functions that are of agricultural and economical importance (Baker & Hunt 1981). Three of these functions include: the control of water exchange with the atmosphere (Schönherr & Schmidt 1979), a barrier protecting plants against ingress by pathogens (Kolattukudy 1985), and it represents the first barrier to penetration of foliar applied compounds. Of these, the main function ascribed to the cuticle is to minimise water loss from plants when stomata are closed (Chamel *et al.* 1991). Dynamic aspects of water vapour loss from leaves have mostly been based on variable stomatal apertures (Zeiger *et al.* 1987) with cuticular resistance usually assumed to be constant (Parkhurst 1986) or negligible (Cowan 1982). During the last decade, however, it has became clear that these assumptions are questionable (Santrucek & Slavik 1990). The hydraulic conductance of the cuticle is especially relevant when estimating rates of transpiration and diffusive water vapour conductance of leaves (Weyers & Meidner 1990).

The importance of cell walls, both in terms of their usefulness to man and in the carbon economy of the biosphere, should also not be overlooked (Goodwin & Mercer 1983). The detailed structure of the cell wall must first be known before we can understand the role that the wall plays as a phase into which and through which transport occurs in absorption by cells and its translocation across tissues (Lüttige & Higinbotham 1979). The general structure of the primary cell wall is composed of cellulose microfibrils embedded in an amorphous mixture of polysaccharides and glycoproteins. Although this structural model appears to be accurate, it lacks detail (Darvill *et al.* 1980). A more detailed description of the primary cell wall will eventually include the following: (1) isolation and identification of each of the individual macromolecular components of the cell wall; (2) determination of the primary structure of each of these macromolecules; (3) determination of the three-dimensional structure of these macromolecules; (4) determination of how and where these macromolecules are biosynthesized within the plant cells; (5) determination of how these macromolecules are attached to one another or how they are interrelated; (6) determination of how the interrelated macromolecules are distributed throughout the thickness and the length of the wall; and, (7) determination of how newly synthesized macromolecules are inserted into the wall and how the wall grows (Darvill *et al.* 1980). The cell wall is biologically important for two reasons. Firstly, it largely determines the morphology and the functioning of the cell. Secondly, since it forms the envelope of the cell, it may be directly involved in regulating cell expansion (Darvill *et al.* 1980; Goodwin & Mercer 1983). The wall also represents a structural barrier to some molecules and to invading pests (Lüttige & Higinbotham 1979). Walls are involved in metabolic events such as pre-absorptive processing of nutrients and post-secretary processing of wall molecules. The ability of the wall to activate plant defense mechanisms and mimic plant hormones suggests that wall components possess enormous information potential and that cell walls are able to play an active role in cell-cell communication (Bacic *et al.* 1988). Thus, understanding the molecular detail of the biosynthesis and assembly of the plant cell wall and its components is essential for an accurate description of the physiology of plant growth (Delmer & Stone 1988).

In the quest for answers on the plant cuticle and cell wall ultrastructure three points should always be kept in mind. (I) The cell wall and cuticle of plants are heterogeneous in structure and care should be taken not to over-simplify and generalise the ultrastructure of cuticles and cell walls of different plant species. (II) Plant function can ultimately be understood on the basis of the principles of physics and chemistry (Salisbury & Ross 1992). (III) In living organisms, structure and function are intimately wedded (Salisbury & Ross 1992). All these aspects should be taken into account to form an understanding of the functional aspects of the plant cuticle and cell wall. In the past researchers often studied either the cuticle or cell wall in isolation, while these composite epidermal structures should, in our view, not be studied separately in light of their integrated function and structure.

Previously scanning electron microscopy, has been unable to demonstrate cuticular and cell wall fine structure due to limited resolution. Detailed information is, however, essential to understand why between cultivars of the same species epidermal transpiration differs (Van Rensburg & Peacock 1998). The present investigation was conducted in order to extend experimental evidence, on transcuticular water movement. To our knowledge this is the first study of the three dimensional ultrastructure of the

plant cuticle and cell wall using high resolution field emission scanning electron microscopy.

Materials and Methods

Plant material

Four cultivars of *Nicotiana tabacum* L., TL33, CDL28, GS46 and Elsoma (listed in sequence of increasing drought tolerance), were used. Seed was germinated in soil in pots. The young seedlings developed under glasshouse conditions with optimal water application. Before the onset of the experiment the plants were moved to a growth room and allowed to acclimatise for 98 hours. The growth chambers were lit for 13 hours at 25°C followed by an 11-hour dark period at 16°C. Experiments started when plants were approximately 90 days old (for more detail see Van Rensburg *et al.* 1993). The sixth youngest leaf representing a mature, nearly fully expanded leaf before the onset of senescence (Van Rensburg *et al.* 1993), was used during sampling. Samples were cut with a sharp razor blade from the center of the lamina between the main vein and the margin, avoiding large lateral veins. The adaxial side of the leaf was studied for all the methods used.

Transmission electron microscopy

Samples 1 mm^2 were cut from the sixth leaf, in the same position as described above. The following fixation schedules were carried out at room temperature, unless stated otherwise. All material was dehydrated in an acetone series followed by embedding in epoxy resin (Spurr 1969). (1) Primary fixation in 2% OsO_4 + 4% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 1 hour at 4°C, post-fixation in 2% OsO_4 in the same buffer for 2 hours at 4°C (Franke *et al.* 1969). Sections were contrasted with 5% aqueous uranyl acetate for 40 minutes and lead citrate (Reynolds 1963) for 20 minutes. (2) Primary fixation in 2% OsO_4 vapour for 3 hours, post-fixation in aqueous 2% OsO_4 for 2 hours followed by contrasting with 5% aqueous uranyl acetate for 20 minutes and lead citrate for 10 minutes. (3) Primary fixation in 2.5% formaldehyde (freshly prepared from paraformaldehyde) + 2% glutaraldehyde + 1.5 mM calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hours (Pacini *et al.* 1992), post-fixation in 0.5% OsO_4 for 1 hour and contrasting with 5% aqueous uranyl acetate for 20 minutes and lead citrate for 10 minutes. The three methods were selected from Kruger *et al.* (1996). Different methods reveal different structures of the epidermis which will be compared to the FE-SEM work. All sections were made with a Reichert-Jung Ultracut E microtome using glass knives and examined with a Phillips CM10 transmission electron microscope operated at 100 kV.

Scanning electron microscopy

Plunge freezing

Leaf samples 6 mm^2 were cut from the leaf as described above and mounted upright on a graphite stub in carbon dag (Leit C, Neubauer Chemikalien). The stubs were made from spectrographic graphite rods normally used in high vacuum coating systems. The rod was turned to a diameter of 5 mm with a lathe. Disks, 1 mm thick were cut from the rod and polished to a thickness of 0.9 mm. A smooth surface was obtained by a final polish on smooth paper. Before use, stubs were cleaned in acetone followed by diethylether in an ultrasonic bath. The stub with specimen was plunge frozen in liquid propane at -180°C with a Reichert KF 80-plunge freezer. The time between mounting the specimen and plunging it in propane was 10 seconds. The stub was transferred under liquid nitrogen into a slot in a copper block where the upper part of the tissue pieces were fractured with a sharp razor blade. Tissue for cross sections was usually fractured level with the top surface of the carbon dag. This resulted in tissue fragments not higher than 0.5 mm above the stub surface. The height of the specimen was kept to a minimum because of limited space in the specimen chamber associated with an in-lens type of microscope. This type of microscope also permits high-resolution imaging if a thin metal coating is used. Charging is also reduced when the specimen surface is in close contact with the conductive carbon dag.

The copper block and tissue were kept immersed in LN_2 in a plastic dish. The copper block (63 × 63 × 15 mm) was fitted with a thermocouple and heater. The surface of the block was kept lower than the rim of the plastic dish in order to keep the material in a nitrogen atmosphere to prevent moisture condensation on the tissue. The plastic dish and copper block were transferred to a Fisons high vacuum unit and evacuated immediately. The temperature of the copper block was typically below -130°C when the vacuum reached 1×10^{-2} Torr. The vacuum remained at this value until all nitrogen (frozen) was sublimed from the plastic container. After about 1 hour the vacuum improved to 1×10^{-3} Torr and the temperature rose to -100°C. Freeze-drying was done overnight. After ± 15 hours the vacuum was 1×10^{-4} Torr and the temperature of the copper block was 0°C. The block was then heated to 25°C before releasing the vacuum. Some samples were washed in both 100% chloroform and 100% methanol for 10 seconds before plunge freezing, to determine the influence of these solutes on the leaf surface, especially with regard to the epicuticular wax.

Nitrogen freezing

Samples 6 mm^2 were cut from the leaf and washed in 100% acetone for 15 minutes. Nitrogen freezing was applied to determine the effect of acetone on the cuticle and removal of epicuticular wax. Samples were mounted in carbon dag on a graphite stub and frozen in LN_2. The tissue was fractured level with the top surface of the carbon dag and then left to air dry.

Isolation of the epidermis

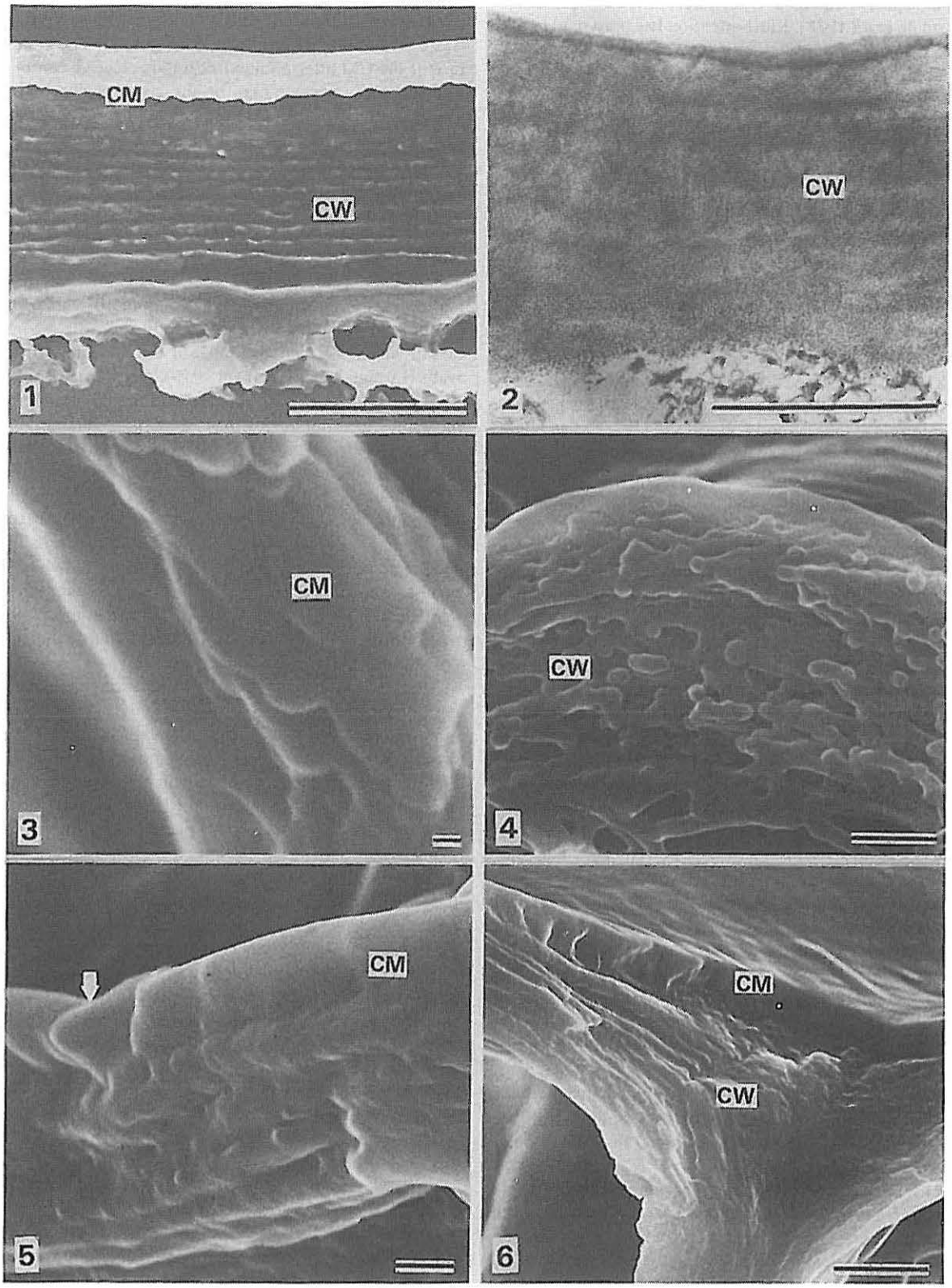
Leaf disks of 5 mm diameter were enzymatically isolated by incubation in 2% (w/v) pectinase, buffered at pH 3.6 with 0.2 M sodium acetate. After removal of the underlying mesophyll cell walls for 48 hours at 37°C, the epidermis was recovered, washed in distilled water, and air dried in the copper loop (4 mm diameter) used to recover the epidermis from the water. After drying, the epidermis was cut from the loop, mounted in carbon dag on stubs and left overnight in a dessicator. This dried mount was trimmed to the level of the carbon dag with a sharp razor blade.

Removal of epoxy resin from sections

Fixation schedule 2 was used for this method as described for TEM. Cross-sections of resin embedded (Spurr 1969) leaf material (*ca.* 100 nm thick) were made with a Reichert-Jung Ultracut E microtome using a diamond knife. Sections were mounted on coverslips and dried on a hot globular (60°C) for 1 hour. The treating solution was prepared as follows: Stock solution contained: 18-crown-16, 0.13 g (Sigma Chemical Co.,Cat. No. 123H7702), DMSO, 9.9 ml and 0.1 ml distilled water. To prepare the working solution, 30% (w/w) methanolic potassium methoxide (0.3 ml) was added to 10 ml of stock solution (Iwadare *et al.* 1990). Since the activity of the working solution decreases rapidly with storage, working solutions were freshly prepared each day. Treatments with the resin removing solution were conducted for 5–60 minutes at room temperature. Slides were thoroughly rinsed with distilled water and left to air dry.

Coating and sample viewing

Samples were coated with chromium (*ca.* 3–4 nm thick) in a Gatan Ion Beam coater model 681. During coating samples were rocked (0–15°) and rotated continuously. For backscatter electron imaging, samples were first coated (*ca.* 3 nm thick) unidirectionally with chromium at an angle of 45° and then with carbon, using the rocking and rotating parameters as described above. Material was viewed with a JEOL F6000 inlens field emission scanning electron microscope. Before the use of the JEOL F6000 SEM a JEOL 840 SEM was used to find specimens on the coverslips after resin was



Figures 1–6 **1**. Resin was removed from ultra-thin sections with 18-crown-16 and viewed with the FE-SEM. The cuticular membrane (CM) is not distinguished into the cuticle proper and cuticular layer. The cell wall (CW) reveals a stratified structure. Scale bar = 1 μm. **2**. The cell wall (CW) displays a faintly stratiform structure viewed with TEM. The specimen was prepared with a primary fixation of 2% OsO_4 vapour and post-fixation aqueous 2% OsO_4 embedded in epoxy resin. Scale bar = 1 μm. **3**. Epoxy resin was removed from ultra-thin sections with 18-crown-16 and viewed with the FE-SEM. The cuticular membrane (CM) shows a globular structure at high magnification. Scale bar = 10 nm. **4**. The cell wall (CW) reveals a reticulate structure after resin was removed. Scale bar = 100 nm. **5**. Material was washed with 100% chloroform and methanol and plunge frozen. The globular structure (arrow) of the cuticular membrane (CM) was discernible. Scale bar = 100 nm. **6**. The specimem was washed in 100% acetone and frozen in LN_2 The globular structure of the cuticular membrane (CM) is again evident. The CM covers the cell wall (CW) of two epidermal cells of tobacco. Scale bar = 1 μm.
removed.

Results and Discussion

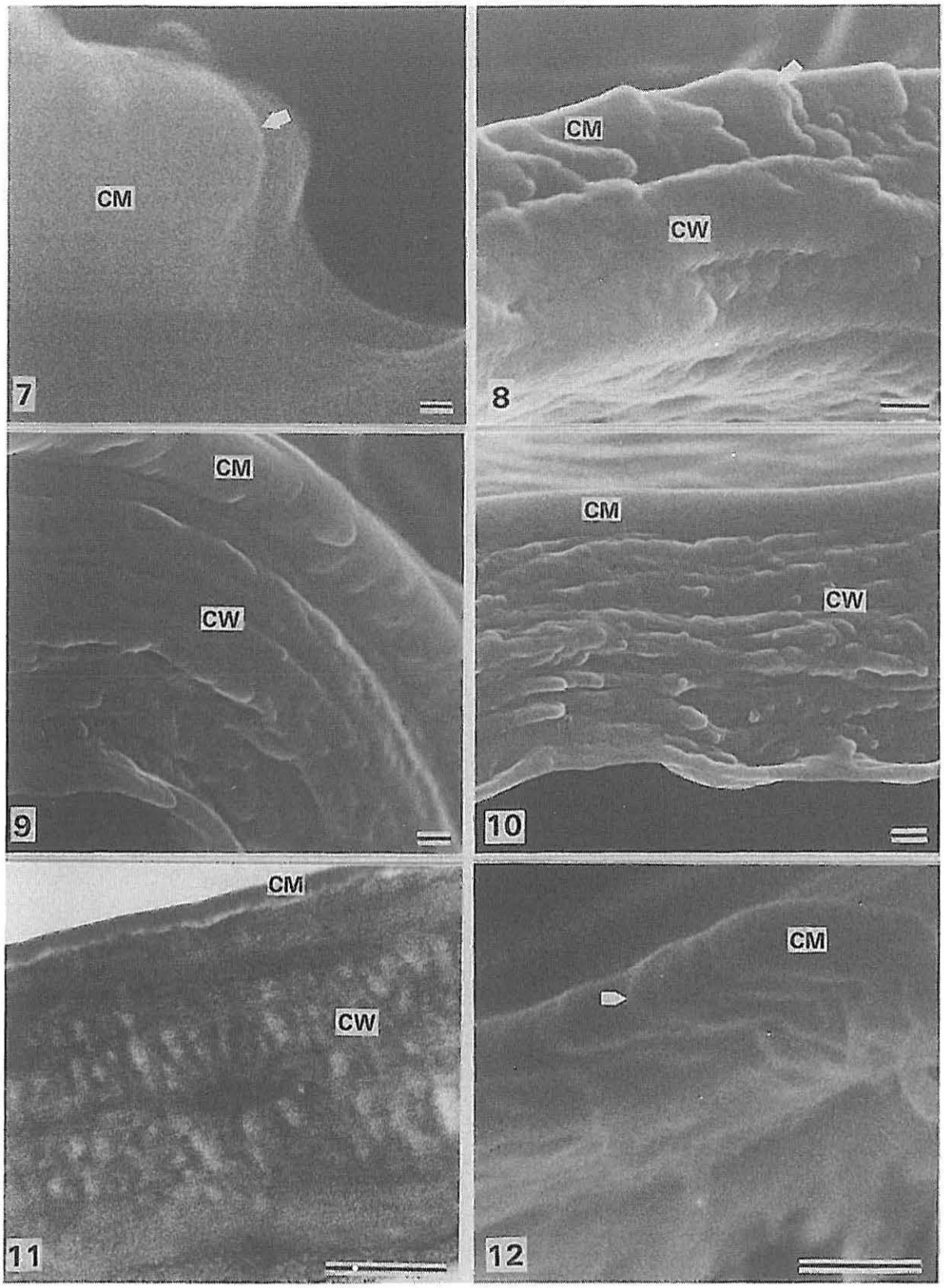
Previous research on the tobacco cuticle has focused mainly on the chemical aspects (Chang & Grunwald 1980; Severson *et al.* 1984; Arrendale *et al.* 1988). Little attention has been paid to the structure of leaf cuticles of mesophytic crop plants such as *Nicotiana tabacum* L., and its relationship to water stress resistance. One of the first studies on the ultrastructure of the cuticle of *N. tabacum* leaves and an evaluation of different preparation techniques for TEM studies of tobacco leaf cuticles, was that by Kruger *et al.* (1996). Their study showed that the leaves of *N. tabacum* exhibit a typical bi-layered cuticular membrane (CM), composed of a thin outer cuticle proper (CP) and a thicker cuticular layer (CL). The CP consisted of electron-opaque lamellae alternating with electron-lucent lamellae, while a reticulate pattern was discernible in the CL. The cellin wall (CW) appeared electron opaque after contrasting with uranyl acetate and lead citrate (Krüger *et al.* 1996). The terminology as proposed by Sitte and Rennier (1963), Wattendorff and Holloway (1980) and Holloway (1982) is used to describe the fine structure of *N. tabacum* cuticle for the transmission electron microscope work. The basic structural concepts of the CM and CW reflected by TEM and light microscopy (LM) are applied to describe the gross morphology of the epidermis wall studied with FE-SEM.

The ultra-thin epoxy sections, from which resin was removed, revealed the basic structure of CM and CW in the FE-SEM investigation. However, the CM could not be distinguished into the CP and CL (Figure 1) as was the case with TEM. The CW was distinctly stratified (Figure 1). The stratification could only be faintly distinguished in the CW studied with TEM (Figure 2). Differences between the TEM and the FE-SEM observations became evident when studying the thin resin embedded sections. Silver and gold sections were used both for TEM and FE-SEM. The only difference in preparation was contrasting of the sections for TEM and removal of resin for FE-SEM. At high magnification the CM appeared amorphous with a globular structure visible (Figure 3) and the cell wall revealed a reticulate structure (Figure 4). Previous results indicated that washing tobacco leaves with 100% acetone, chloroform and methanol removed both intra- and epicuticular waxes (Chu & Tso 1968; Chang & Grunwald 1980; Severson *et al.* 1981; Reed 1982; Severson *et al.* 1984; Arrendale *et al.* 1988). In this investigation the fresh leaf material was washed in 100% chloroform and then in 100% methanol and prepared with plunge freezing. Yet, after this drastic pretreatment the CM was still discernible with the globular structure (Figure 5). The same results were obtained when fresh leaf material was washed with 100% acetone and frozen in LN_2 (Figure 6). As acetone is an effective dehydration agent, there ought not to have been much water left in the tissue that could have resulted in ice crystal damage due to the slower freezing rate in LN_2. These harsh methods indicate that the layer described as the CM studied with the FE-SEM is not the epicuticular wax layer and that the globular structure is consistent.

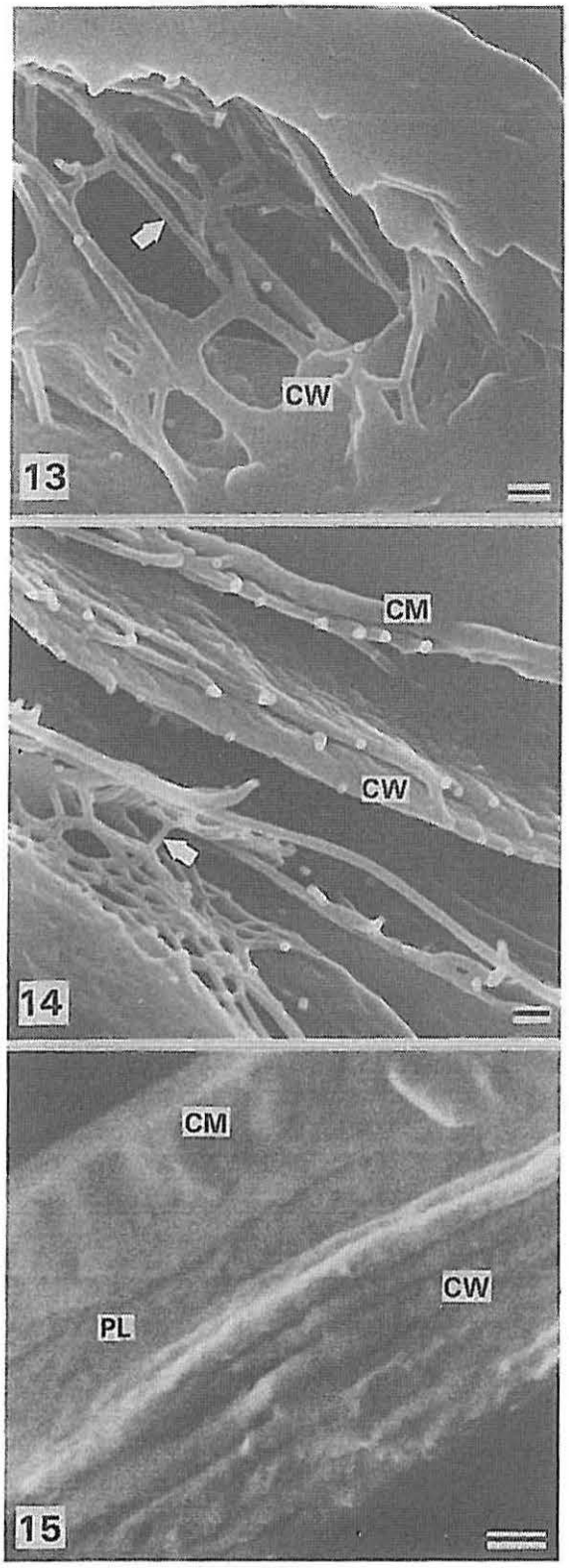
Specimens for electron microscopy can be prepared without any chemical treatment by cryofixation (Ryan 1992). The aim of cryofixation is to arrest and stabilise the structure, processes and composition of biological material by rapid removal of thermal energy (e.g. by freezing) (Hall & Hawes 1991). Plunge freezing was employed in this study as it was important to preserve the epidermal wall as near to the *in vivo* state as possible and to fix specimens by different methods to minimise interpretation of data that may be artifacts caused by only one preparation procedure. The hydrocarbon propane coolant used in the present study freeze at -180°C. This liquid is an efficient coolant mainly because there is a wide range between the freezing and boiling point. The disadvantage of propane is its safety aspects as it is flammable (Ryan 1992; Barnard 1987).

Cryofixation of fresh leaf tissue gave similar results to those observed with the resin embedded sections for TEM and FE-SEM. The CM and CW were discernible but again, as for the resin embedded sections studied with the FE-SEM no CP or CL was evident at high magnification (Figure 7). The globular structure of the CM after plunge freezing is clearly seen in Figure 8. The globular structure may be the consequence of freezing at -180°C as the cuticle may have lost its elasticity. This might have induced a clean fracture in the CM, when the specimens were fractured with a sharp razor blade after freezing. As an hypothesis to explain the fracture plane, we suggest there could be a resemblance in the spatial size of the globular structure and the fibrils in the CL. Thus the fibrillar structure of the CL could be the plane where fracturing occurred after freezing. The CW again revealed a stratified appearance (Figure 9), as also observed in Figures 1 and 2. This is more prominent in Figure 10 where microfibrils protrude from the CW. These microfibrils had a diameter of *ca.* 30 nm which corresponds to that of cellulose microfibrils of cell walls (Lüttige & Higinbotham 1979). According to Salisbury and Ross (1992), about 30 to 40 pairs of long, unbranched cellulose molecules form the microfibril, while Lüttige and Higinbotham (1979) state that about 40 up to 300 cellulose molecules are joined together in the microfibrils. If the small spots discernible in the CW (Figure 11) with TEM work (see Kruger *et al.* 1996) are cellulose molecules (*ca.* 5 nm diameter for tobacco), it could mean that there are 36 cellulose molecules in microfibrils of tobacco. This hypothesis is compatible with both the TEM and FE-SEM results, and shows how these techniques complement one another. The FE-SEM work revealed the gross morphology of the microfibrils while the TEM showed the cellulose molecules in the cross sections of the cell wall.

The globular structure could also be distinguished with the epidermis enzymatically isolated (Figure 12). A globular structure manifested even though the specimen was not frozen but air-dried. In this regard, it is interesting to note that Viougeas *et al.* (1995) concluded that isolated cuticles presented no structural alteration compared to non-isolated cuticles and, therefore, isolated cuticles could be used for permeability studies. A possible explanation could be that the cuticle is elastic and tears like rubber in the same plane as the fracture planes of frozen specimens when they are trimmed. However, the cell wall appeared at low magnification to have a flaky appearance (Figure 13) as if the strata pulled away from each other. Figure 14 shows a clear presentation of the cell wall morphology. The stratification is discernible with some strata showing a network of microfibrils while others reveal the matrix in which the microfibrils are embedded. The microfibrils in Figures 13 and 14 are *ca.* 30 nm in diameter, that coincides with that of cellulose microfibrils (Lüttige & Higinbotham 1979). The cell wall materials of tobacco contain 34% pectin, 21.6% protein, 18.7% cellulose, 11.4% hemicellulose and 4.1% lignin (Ruben & Bokelman 1989). The matrix consists of hemicelluloses and pectin (Lüttige & Higinbotham 1979; Goodwin & Mercer 1983). The matrix also includes glycoproteins, with the protein fraction particularly rich in the amino acid hydroxyproline (Lüttige & Higinbotham 1979; Darvill *et al.* 1980). Pectin is the major component found in the primary cell walls of dicotyledonous (Ruben & Bokelman 1989; Darvill *et al.* 1980) with a galacturonic acid content of *ca.* 80% for tobacco pectin (Ruben & Bokelman 1989). It is not clear from the literature whether the pectin layer is an integral part of the cuticular layer, an additional layer of the CM, or a region of the epidermal wall (Holloway 1982). With the FE-SEM no layer could be identified as being the definite pectin layer. Images of plunge frozen material, imaged with backscattered electrons, might reveal a pectin layer visible between the CM and CW (Figure 15) indicating that this layer is either part of the CM or CW. Therefore, the



Figures 7–12 7. Plunge freezing showing the globular structure (arrow) of the cuticular membrane (CM) at high magnification, with no CP or CL visible. Scale bar = 10 nm. **8**. The cell wall (CW) and cuticular membrane (CM) with the globular structure (arrow) are visible after plunge freezing. Scale bar = 100 nm. **9**. Plunge freezing reveals a stratified cell wall (CW) and cuticular membrane (CM). Scale bar = 100 nm. **10**. The cellulose microfibrils (*ca.* 30nm) are visible in the CW. Scale bar = 100 nm. **11**. The method of Pacini *et al.* (1992) was used to prepare the specimen and post-fixation 5% OsO_4 for TEM. The cell wall (CW) reveals the stratiform structure with electron-lucent spots, which may represent cellulose microfibrils. Scale bar = 500 nm. **12**. A globular structure (arrow) in the cuticular membrane (CM) is visible. Scale bar = 100 nm.



Figures 13–15 **13**. The cell wall (CW) strata which pulled away from one another during enzymatic isolation display possible cellulose microfibrils (arrow). Scale bar = 100 nm. **14**. The CW is differentiated into strata revealing the possible cellulose microfibrils (arrow), displaying an open network without matrix, while the first few strata reveal microfibrils with matrix. Scale bar = 100 nm. **15**. This specimen was plunge frozen and imaged with backscatter electrons in the FE-SEM. The cuticular membrane (CM) and cell wall (CW) can be distinguished. Is it possible that the layer between the CM and CW might be the pectin 'layer'(PL)? Scale bar = 100 nm.

distribution and function of pectin are no longer compatible with the idea that it is simply a gel (Roberts 1990). Future FE-SEM work on pectin may reveal the pectin morphology. Recent work on the pectin layer has established that boron is associated with cell wall pectins and the contingent effects of boron on cell wall extensibility suggest that boron plays a critical, although a poorly defined, role in the cell wall structure of higher plants (Hu & Brown 1994).

As with the TEM, no epicuticular wax was discernible with any of the methods used in this study. Surface wax is present on species such as *N. tabacum* that has been classified as non-wax bearing (Hall 1967). In many species the wax occurs as a relatively thin structureless sheet (Hall 1967). Baker (1982) stated that the aerial surfaces of all higher plants carry a partial or continuous coverage of amorphous wax. Although epicuticular wax of tobacco is not visible, chemical analyses indicate that the epicuticular waxes exist, with major wax components being C_{25}–C_{36} normal and branched chain aliphatic hydrocarbons, diterpenes, sucrose esters and wax esters (Severson *et al.* 1984; Arrendale *et al.* 1988). Thus, even though chemical analyses indicate that tobacco epicuticular wax exists on the leaf surfaces, the high resolution FE-SEM could not reveal this thin wax layer in this study.

Conclusion

The TEM has provided a wealth of new structural information about the plant CM and CW, but it has not always confirmed some of its most characteristic features in the light microscope (Holloway 1982). The FE-SEM work in which different methods were applied revealed a globular structured CM, which are not wax layers, and a stratiform CW, with microfibrils of *ca.* 30 nm in diameter. The position of pectin in the cell wall could not yet be determined in this study with the use of the FE-SEM. Pectin may have an important structural function in the cell wall and may facilitate permeability and is thus no longer compatible with the idea that it is simply a gel. Future studies are necessary to determine the position and function of pectin in the cell wall. The results obtained with the FE-SEM revealed three-dimensional structural information about the CM and CW not previously observed, which could have a bearing on the water transport properties of the cuticle and cell wall, however, more detailed information is necessary on the epidermal wall ultrastructure to explain epidermal transpiration.

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